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Award Number: W81XWH-14-1-0441

TITLE: Identifying Determinants of PARP Inhibitor Sensitivity in Ovarian Cancer

PRINCIPAL INVESTIGATOR: Neil Johnson, Ph.D.

CONTRACTING ORGANIZATION:

Institute for Cancer Research
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REPORT DATE: October, 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

Approved for Public Release; Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* October 2015	2. REPORT TYPE Annual	3 DATES COVERED 15Sep2014-14Sep2015

4. TITLE AND SUBTITLE
Identifying Determinants of PARP Inhibitor Sensitivity in Ovarian Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0441

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We hypothesize that a range of common ovarian cancer predisposing germ-line BRCA1 gene mutations produce semi-functional proteins that are capable of providing PARP inhibitor resistance. Gene mutations that result in the activation of DNA end resection are likely to be required for restoration of HR DNA repair in this setting. Additionally, genetic events that stabilize mutant BRCA1 proteins may be required to avoid proteasome-mediated degradation. Our objectives are to define the BRCA1 peptide region minimally required for PARP inhibitor resistance, and discover genetic alterations that activate DNA end resection as well as mutant BRCA1 protein stabilization in ovarian carcinomas. The expression of mutant BRCA1 or novel proteins identified to be important for drug resistance will be assessed for their ability to be used as biomarkers of PARP inhibitor or platinum response. Protein expression in tumors will be assessed for their potential to serve as biomarkers that predict PARP inhibitor or platinum response.

15. SUBJECT TERMS
Ovarian cancer, BRCA1, RAD51, PARP inhibitors, platinum, biomarkers, drug resistance

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	14	19b. TELEPHONE NUMBER *(Include area code)*
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Cells that are deficient in homologous recombination (HR) DNA repair, such as those lacking functional BRCA1 are highly sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors. Ovarian cancer patients that harbored germ-line *BRCA1* mutations treated with PARP inhibitors exhibited meaningful responses in early phase clinical trials. However, emerging clinical trial data indicates that PARP inhibitor therapy may benefit only a subset of *BRCA1* mutation carriers. We hypothesize that a range of common ovarian cancer predisposing germ-line *BRCA1* gene mutations produce semi-functional proteins that are capable of providing PARP inhibitor resistance. Our preliminary data suggests that PARP inhibitor selection pressure results in genetic adaptations that enable cells to utilize severely truncated BRCA1 proteins for RAD51 loading and HR repair. Our objectives are to define the BRCA1 peptide region minimally required for PARP inhibitor resistance, and discover genetic alterations that activate DNA end resection as well as mutant *BRCA1* protein stabilization in ovarian carcinomas. The expression of mutant BRCA1 or novel proteins identified to be important for drug resistance will be assessed for their ability to be used as biomarkers of PARP inhibitor or platinum response. *Specific Aims*. 1: Identify the region of mutant BRCA1 protein critical for PARP inhibitor resistance; 2: Identify genetic alterations essential for PARP inhibitor resistance; 3: Determine the ability of identified genetic aberrations to serve as predictive biomarkers. Protein expression in tumors will be assessed for their potential to serve as biomarkers that predict PARP inhibitor or platinum response.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).
   Ovarian cancer, BRCA1, RAD51, PARP inhibitors, platinum, biomarkers, drug resistance.

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Major Task 1: Determine the minimum region of BRCA1 protein required for PARP inhibitor resistance.
Milestone(s) Achieved: Drug resistant cell lines are derived that express different types of mutant BRCA1 proteins. Target date for completion: 12 months (from start of award). Current status: 80% completed. Expected completion date: 16 months.

Major Task 2: Determine the minimum region of BRCA1 protein required for RAD51 focus formation.
Milestone(s) Achieved: The minimum region of mutant BRCA1 protein is identified that can contribute to RAD51 loading. Target date for completion: 16 months (from start of award). Current status: 80% completed. Expected completion date: 16 months.

Major Task 3: Generate whole exome sequencing and gene expression data sets for PARP inhibitor sensitive and resistant cell lines.
Milestone(s) Achieved: Identification of genes that are mutated or differentially expressed in PARP inhibitor resistant cells. Target date for completion: 30 months (from start of award). Current status: 25% completed. Expected completion date: 30 months.

Major Task 4: Validate identified genes as resistance causing through RNAi and cDNA overexpression techniques.
Milestone(s) Achieved: Validation of genes that are mutated or differentially expressed and result in PARP inhibitor resistance. Target date for completion: 36 months (from start of award). Current status: 0% completed. Expected completion date: 36 months.

Major Task 5: Obtain IRB approval for assessing tumor DNA and assembling tumor microarrays.

Milestone(s) Achieved: IRB protocol approved. Target date for completion: 40 months (from start of award). Current status: 100% completed. Expected completion date: completed.

Major Task 6: Analyze tumor DNA for mutations.
Milestone(s) Achieved: Identification of gene mutation or protein expression changes in ovarian tumors. Target date for completion: 55 months (from start of award). Current status: 0% completed. Expected completion date: 55 months.

Major Task 7: Determine the ability of potential biomarkers to predict therapeutic outcome.
Milestone(s) Achieved: Biomarkers are ready for application in clinical trials of BRCA1 mutant ovarian cancer and data is published in peer-reviewed journals. Target date for completion: 60 months (from start of award). Current status: 0% completed. Expected completion date: 60 months.

What was accomplished under these goals?

Major Task 1: Determine the minimum region of BRCA1 protein required for PARP inhibitor resistance.

BRCA1 directly interacts with RAD51 at BRCA1 amino acid residues 758-1064. Additionally, BRCA1 can indirectly interact with RAD51 through the formation of a BRCA1-PALB2-BRCA2-RAD51 protein complex, mediated by direct binding of BRCA1 amino acid residues 1393-1424 with PALB2. It is not clear if the direct BRCA1-RAD51 or the indirect BRCA1-PALB2-BRCA2-RAD51 complex is most crucial for RAD51 loading onto DNA breaks. We will have generated a series of truncated BRCA1 proteins to elucidate the region of BRCA1 protein critical for RAD51 focus formation and PARP inhibitor resistance.

We initially made BRCA1 cDNA constructs that lacked exons 16-24 as well as exons 12-24, these constructs are referred to as BRCA1Δ16-24 and BRCA1Δ12-24, respectively. BRCA1Δ16-24 lacks the BRCT domains of BRCA1 but retains both the direct and indirect RAD51 interaction motifs. BRCA1Δ12-24 lacks the BRCT domains of BRCA1 as well as the indirect PALB2-BRCA2-RAD51 interaction domain but retains the proposed direct RAD51 interaction motif. MDA-MB-436 cells contain a *BRCA1* 5396+1G>A mutation resulting in a C-terminal BRCT domain deficient protein. These cells are exquisitely sensitive to PARP inhibitor treatment and have undetectable levels of RAD51 foci formation. Therefore we used these to generate isogenic cell lines expressing our BRCA1 constructs of interest. MDA-MB-436 cells were infected with lentivirus containing GFP-IRES-HA-BRCA1 mutations or mCherry control constructs and cell populations enriched for GFP positive cells using flow cytometeric sorting. For all experiments, MDA-MB-436 cells expressing mCherry or wild-type (Full-length) HA-BRCA1 were used as negative and positive controls. For functional analyses, we cultured MDA-MB-436 cells expressing mutant BRCA1 proteins in the presence of 20 nM rucaparib until drug resistant clones emerged. Drug resistant clones emerging from 2 independent sets of 6 well plates were counted and the ability of BRCA1 truncated exogenous proteins to induce drug resistance assessed (Figure 1). We found that BRCA1Δ16-24 provided drug resistance, however BRCA1Δ12-24 did not induce any degree of drug resistance and behaved similarly to mCherry negative control expressing cells.

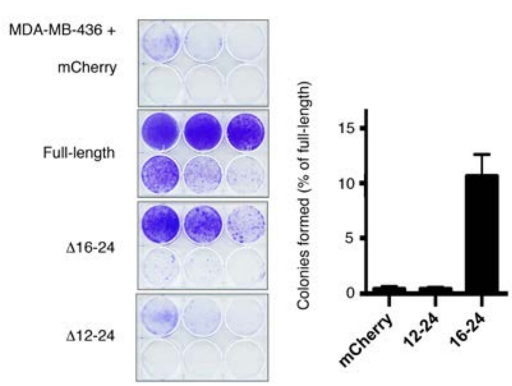

Figure 1. BRCA1 truncated from exon 16-24 can induce PARP inhibitor resistance. MDA-MB-436 cells overexpressing mCherry (negative control), full-length BRCA1, exons 16-24 or 12-24 deleted were assessed for their ability to grow through the presence of 20 nM rucaparib treatment in long term culture. Cells were seeded at increasing densities in 6 well plates and the number of colonies forming was determined and calculated as a percentage of the wild-type expressing cells.

We next hypothesized that the PALB2-BRCA2-RAD51 interacting region was the critical region of BRCA1 that was required to induce PARP inhibitor resistance that was absent from the BRCA1Δ12-24 construct but present in the BRCA1Δ16-24 construct. To test this possibility, we generated a missense mutation L1407P in the BRCA1 16-24 as well as the full length BRCA1 construct. This mutation has been shown previously to prevent BRCA1-PALB2 interaction. We carried out the same assay as described in Figure 1 to determine the ability of full length as well as 16-24 constructs with and without PALB2 interacting missense mutations to provide long term resistance to rucaparib treatment. We found that cells expressing either full length or 16-24 BRCA1 that harbored the PALB2 interaction domain mutation, had a significantly diminished capacity to induce rucaparib resistance (Figure 2). Therefore we believe that the critical region of BRCA1 required for rucaparib resistance encompasses the region required for PALB2 interaction that creates an indirect BRCA1-PALB2-BRCA2-RAD51 complex.

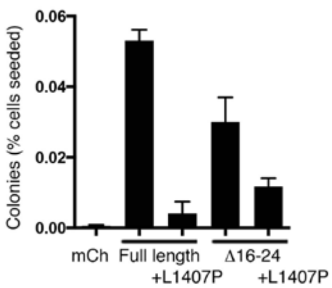

Figure 2. The BRCA1-PALB2 interaction is critical for rucaparib resistance. MDA-MB-436 cells overexpressing mCherry (negative control), full-length BRCA1, full-length BRCA1 with the L1407P missense mutation, exons 16-24 or 16-24 with the L1407P missense mutation were assessed for their ability to grow through the presence of 20 nM rucaparib treatment in long term culture. Cells were seeded at increasing densities in 6 well plates and the number of colonies forming was determined and calculated as a percentage of cells seeded per well.

Major Task 2: Determine the minimum region of BRCA1 protein required for RAD51 focus formation.

To determine how the above described mutations impact RAD51 loading, we utilized cell lines described above and measured RAD51 foci formation by immunofluorescence. Similar to rucaparib resistance assays, the BRCA1Δ16-24 construct provided partial RAD51 foci rescue whereas BRCA1Δ12-24 did not have any RAD51 foci rescue. Furthermore, when the PALB2 interaction domain was mutated to the L1407P mutation, the RAD51 foci rescue was completely abolished. Interestingly, when we treated BRCA1Δ16-24 expressing cells with siRNA targeting 53BP1, the amount of RAD51 foci positive cells was dramatically increased more similar to full-length protein levels. In contrast when PALB2 binding site was mutated, the RAD51 increased by 53BP1 siRNA was abolished. These data indicate that the BRCA1Δ16-24 protein is hypomorphic, and can induce RAD51 foci and PARP inhibitor rescue though its retained ability to interact with and load PALB2-BRCA2-RAD51. BRCA1 hypomorphic proteins lacking the BRCT domains likely depend on additioanl genetic events such as 53BP1 depletion that result in enhanced RAD51 loading (Figure 3).

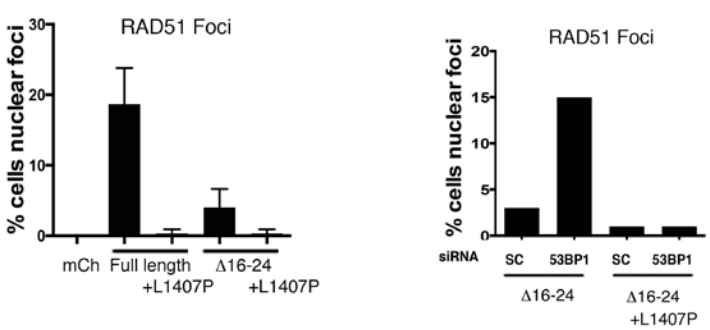

Figure 3. Immunofluoresence assessment of RAD51 foci formation. MDA-MB-436 cells overexpressing mCherry (negative control), full-length BRCA1, full-length BRCA1 with the L1407P missense mutation, exons 16-24 or 16-24 with the L1407P missense mutation were assessed for RAD51 foci formation after 10 Gy irradiation (left). BRCA1 exons 16-24 or 16-24 with the L1407P missense mutation were assessed for RAD51 foci after scrambled or 53BP1siRNA treatment (right). Mutation of the PALB2 domain abolished RAD51 foci.

To confirm the exact region that could contribute to hypomorphic BRCA1 activity, we will generate stop codons before and after the PALB2 interaction domain to define the patient mutation locations that could generate semi-functional or non-functional BRCA1 proteins. We are also beginning to work on additional tasks stated in the time line.

What opportunities for training and professional development has the project provided?

During the first year of this award, I have taken part in the following training activities:

- Discussed progress with Drs. Boyd and Golemis.
- Attended Junior Faculty Mentoring Meetings.
- Presented research at the monthly Faculty Seminar.

During the first year of this award, I have taken part in the following professional development activities:

- I frequently attend FCCC Gynecologic Cancer Meetings
- I attended the Society of Gynecologic Oncology Annual Meeting in Chicago.
- I attended the Ovarian Cancer Academy Meeting in Chicago.
- I attended the AACR in Philadelphia.
- I attended and give a lecture at the Basser Center for BRCA annual symposium, Philadelphia.

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, we will generate whole exome sequencing and gene expression data sets for PARP inhibitor sensitive and resistant cell lines. The goal is to identify genes that work with hypomorphic BRCA1 proteins, such as 53BP1, to promote DNA repair and PARP inhibitor resistance.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change
Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report.

Changes that had a significant impact on expenditures
Dr. Yang is no longer involved on this project. He was replaced by Dr. Karis, whose salary is supported on an NIH Training Grant (T32 CA009035).

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

6. **PRODUCTS:** Nothing to Report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	***N. Johnson, Ph.D.***
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	Neiljohn
Nearest person month worked:	5
Contribution to Project:	Overall administration and guidance of research; Management and training of personnel
Funding Support:	N/A
Name:	***J. Boyd, Ph.D.***
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Dr. Boyd is the mentor on this project. He provides advice, assistance and support.
Funding Support:	*N/A*
Name:	***Y. Wang, Ph.D.***
Project Role:	Postdoc Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Wang contributed to the cell culture and molecular biology experiments.
Funding Support:	N/A
Name:	***J. Krais, Ph.D.***
Project Role:	Posotdoc Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Dr. Krais replaced Dr. Wang on this project. He contributed to the cell culture and molecular biology experiments.
Funding Support:	Salary support by NIH T32
Name:	J. ***Nascon***
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	*Mr. Nascon assists with the* cell culture and molecular biology experiments.
Funding Support:	*N/A*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

- Yes. Dr. Wang was awarded an individual DOD-Pilot and transitioned from this grant effective 7/1/15. Dr. John Krais joined the lab and this project assuming some of Dr. Wang's responsibilities on the project. Other support staff will also be contributing to the projects aims and goals.

- Dr. Johnson's mentor, Dr. Jeffrey Boyd, left Fox Chase for Florida International University in early-July 2015. Dr. Boyd will continue in the capacity of mentor and will issued a subcontract based on his efforts. This subcontract is currently in process.

- Dr. Peri was scheduled to provide 5% of his effort during Year 2 of the grant however Dr. Peri's contributions are charged to the grant as facility chargebacks. Additional analysis if needed from Dr. Peri will be provided through the facility chargeback system.

- *Please see Other Support attached*. Changes from the last reporting period are marked with a line in the right hand margin.

What other organizations were involved as partners?

Organization Name: Florida International University
Location of Organization: Miami, Florida
Partner's contribution to the project: Mentor (Subcontract agreement in process)
- Dr. Boyd devotes 10% effort as a mentor on this project. Funds are also allotted for travel.

Organization Name: Temple University
Location of Organization: Philadelphia, PA
Partner's contribution to the project: Consultant
- Dr. Burger provided advice and guidance on the development of biomarkers that predict tumor PARP inhibitor sensitivity. His role will increase in the upcoming years.

Organization Name: University of Washington
Location of Organization: Seattle, WA
Partner's contribution to the project: Consultant
- Dr. Swisher will provided advice and guidance on the development of on the biomarkers that predict tumor PARP inhibitor sensitivity. Her role will increase in the upcoming years.

8. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** Not Applicable.
   - **QUAD CHARTS:** Not Applicable.

9. **APPENDICES:** Not Applicable.

Other Support

Johnson, Neil

ACTIVE

R21 CA191690 (PI: Johnson) 3/17/2015 - 2/28/2017 2.04 calendar
NIH $108,750
Identifying BRCA1 Protein Variants that Provide Resistance to Therapy
The major goals of this project are: 1) To identify BRCA1 isoforms highly expressed in drug resistant tumors; and 2) To determine the ability of BRCA1 isoforms to provide HR DNA repair and drug resistance.

W81XWH-14-1-0441 (PI: Johnson) 9/15/2014 - 9/14/2019 4.20 calendar
Army $142,051
Identifying Determinants of PARP Inhibitor Sensitivity in Ovarian Cancer
The major goals of this project are: 1) To identify the region of mutant BRCA1 protein critical for PARP inhibitor resistance; 2) To identify genetic alterations essential for PARP inhibitor resistance; and 3) To determine the ability of identified genetic aberrations to serve as predictive biomarkers.

W81XWH-15-1-0197 (PI: Johnson) 7/1/2015 - 6/30/2017 1.80 calendar
Army $147,836
Determine the Impact of Novel BRCA1 Translation Start Sites on Therapy Resistance in Ovarian Cancer
The major goals of this project are: 1) To identify germline BRCA1 mutations capable of generating drug resistance-inducing N-terminal deficient proteins; 2) To characterize the role of N-terminal deficient BRCA1 proteins in the DNA damage response; and 3) To identify small molecules that kill N-terminal deficient BRCA1 protein expressing cells.

CCR12226280 (PI: Johnson) 3/7/2013 - 3/6/2016 3.60 calendar
KOMEN $120,000
Determining the Role of PARP Inhibitors in BRCA1 Mutant Breast Cancer
The major goals of the career development award are: 1) To determine PARP inhibitor resistance mechanisms in BRCA1-mutant breast cancer cell lines; 2) To identify small molecules that sensitize resistant BRCA1-mutant cells to PARP inhibition in vitro; and 3) To determine the ability of novel small molecules to sensitize resistant BRCA1 mutant cells to PARP inhibition in vivo.

OVERLAP
None

Other Support

Boyd, Jeff

ACTIVE

W81XWH-14-1-0441 (PI: Johnson) 9/15/2014 - 9/14/2019 1.20 calendar
Army Partial Salary
Identifying Determinants of PARP Inhibitor Sensitivity in Ovarian Cancer
The major goals of this project are: 1) To identify the region of mutant BRCA1 protein critical for PARP inhibitor resistance; 2) To identify genetic alterations essential for PARP inhibitor resistance; and 3) To determine the ability of identified genetic aberrations to serve as predictive biomarkers.

COMPLETED

P50 CA083638

OVERLAP

None

Other Support

Peri, Suraj

Dr. Peri's efforts are being covered through a facility chargeback.

Other Support

Krais, John

ACTIVE

W81XWH-14-1-0441 (PI: Johnson) 9/15/2014 - 9/14/2019 12.00 calendar
Army No Salary
Identifying Determinants of PARP Inhibitor Sensitivity in Ovarian Cancer
The major goals of this project are: 1) To identify the region of mutant BRCA1 protein critical for PARP inhibitor resistance; 2) To identify genetic alterations essential for PARP inhibitor resistance; and 3) To determine the ability of identified genetic aberrations to serve as predictive biomarkers.

OVERLAP

None

Other Support

Wang, Yifan

Dr. Wang is no longer associated with this project. He has been replaced by Dr. Krais.